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Immunohistochemical evaluation of heat shock proteins in normal and preinvasive lesions of the cervix☆

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Abstract

Heat Shock Proteins (HSPs) are molecular chaperons with multiple functions relating to cellular homeostasis. Primary, HSPs are expressed in response to cellular stresses that may also include carcinogenesis. Patterns of expression have not been extensively evaluated in the multi-step carcinogenesis of cervical cancer (Normal → HPV infection → Cervical Precancer → Cancer). We evaluated the expression of HSP40, HSP60, HSP70, and HSP90 in normal tissues ($N=30$), in cervical intraepithelial neoplasia grade 1 (CIN1)(synonymous with productive HPV infections) ($N=32$), and in CIN3 (cervical precancer)($N=25$) by immunohistochemistry staining (graded 0–3) and compared the results to $p16^{INK4a}$, a biomarker of oncogenic HPV infections and CIN3. We found strong patterns of increased HSP40, HSP60, and HSP70 immunostaining with increasing severity of the lesion in a manner similar to $p16^{INK4a}$ ($P_{Trend}<0.0005$). No difference in staining intensity by grade of lesion was observed for HSP90 ($P_{Trend}=0.8$). Tissue patterns in CIN3 of diffuse immunostaining for HSP40 and HSP70 were analogous to those observed for $p16^{INK4a}$; HSP60 immunostaining appeared more punctate within cells than for other antigens although similar tissue patterns were observed. We conclude that HSP40, HSP60, and HSP70 expressions are up-regulated in response to the development of CIN3 akin to $p16^{INK4a}$ expression.

Keywords: Cervical intraepithelial neoplasia; Human papillomavirus (HPV); Heat shock proteins; HSP40; HSP60; HSP70; HSP90; Immunohistochemistry

1. Introduction

The heat shock or stress response is a highly evolutionarily conserved, adaptive mechanism for cellular survival [1]. In response to endogenous and exogenous stresses, the expression of proteins synonymous with this protective response, including heat shock proteins (HSPs), is altered and typically up-regulated [1,2]. These proteins perform a wide

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range of homeostatic, housekeeping, and cytoprotective functions. Perhaps not surprisingly, the stress response is now implicated as fundamental component of the cellular immune response [2], serving as early non-specific (i.e., innate immune) surveillance and response to non-physiologic conditions such as infection.

There is now evidence that HSP expression may be altered in response to carcinogenesis. HSP60 and HSP90 are reportedly down-regulated in bladder carcinoma [3]. Increased HSP27 expression has been suggested as a biomarker of ovarian cancer prognosis and survival [4,5] and there is a reported increase in the prevalence of HSP90 autoantibodies in late stage ovarian cancer [6], suggesting that HSP90 may also be up-regulated. Increased HSP70 has been reported in ovarian cell lines [7]. Increased HSP70 expression was found to be associated with recurrence in node-negative breast cancer patients [8]. For salivary carcinoma, one immunohistochemistry study found a decrease expression of HSP27, HSP70, HSP90, and HSP110 [9] whereas another study using a proteomics approach found an increase expression of HSP27 and HSP70 [10] in cancer cases versus controls. HSP27 expression is associated with more aggressive and poorly differentiated oral squamous cell carcinoma [11] and with greater proliferation and drug resistance in human renal cell carcinoma [12].

However, there is a lack of data regarding the HSP expression in response to cervical carcinogenesis. There are single reports of increased expression of HSP60 [13] and HSP70 [14] with severity of cervical lesions, and one report of positive HSP70 detection by immunohistochemistry in 73% of cancer cases [15]. It is now widely accepted that sexually-transmitted cervical infections by approximately 15 human papillomavirus types cause virtually all cervical cancer worldwide [16–18]. As the result of rigorous and numerous epidemiologic studies, it is now understood that there are several definable steps in cervical carcinogenesis [19]: Normal → HPV Infection → HPV Viral Persistence → Precancer → Cancer. Thus, changes in HSP expression could result in response to HPV infection and/or progression of HPV infected tissues to precancer and cancer. To address the relationship of HSP expression to early cervical carcinogenesis, we undertook an immunohistochemistry study to examine and compare the expression of HSP40, HSP60, HSP70, and HSP90 in tissue diagnosed as normal, as cervical intraepithelial neoplasia grade 1 (CIN1) (synonymous with productive viral infection), and as CIN3 (cervical precancer).

2. Materials and methods

2.1. Specimens

A convenience sample of paraffin-embedded cervical tissues diagnosed as normal ($N=30$), cervical intraepithelial neoplasia grade 1 (CIN1) ($N=32$), and CIN3 were drawn from tissue archives within the Department of Pathology at the University of Texas Southwestern Medical Center in Dallas, TX. We excluded CIN2 from our study because it is a very poorly reproducible histopathologic diagnosis [20] that likely represents a mixture of CIN1 and CIN3; we excluded cancer cases because we were interested in early carcinogenesis and because of the possibility of disease effects. The study was approved by University of Texas Southwestern Medical Center institution review board and was deemed exempt from review by the NIH (Bethesda, MD). Multiple 4 μm tissue sections were cut from the embedded tissues using a microtome and were used for hematoxylin and eosin (H&E) staining and for immunohistochemistry staining, the latter of which was done in a masked fashion. H&E slides were evaluated for cervical inflammation (no, mild, or severe inflammation), masked to the results of the HSP immunostaining.

2.2. Immunohistochemistry

All immunohitochemical staining was performed at room temperature and carried out using the Dako Autostainer™ (Dako, Carpinteria, CA). The Envision Plus Detection Kit (Dako) and Dako™ Target Retrieval Solution, pH 6.0 (Dako) were used. Optimum primary antibody dilutions were predetermined using known positive control tissues. A known positive control section was included in each run to assure proper staining.

Paraffin sections were cut at 4 μm on a rotary microtome, mounted on positively charged glass slides (Superfrost, Fisher, Pittsburgh, PA), and

baked overnight at 37 °C. Sections were then deparaffinized in xylene and ethanol and subsequently placed in 200 ml Target Retrieval Solution. The buffer was brought to a temperature of 100 °C for 20 min. Sections were then removed and allowed to cool in ambient temperature buffer for 20 min and then rinsed thoroughly in deionized water. In the Dako Autostainer, sections were first treated with 3% H_2O_2 for 5 min to quench endogenous perioxidase and then rinsed. Quenched sections were incubated with primary antibody overnight. After rinsing to remove the primary antibody, sections were incubated for 30 min with Dako EnVision+™ peroxidase-conjugated, anti-Rabbit or anti-Mouse IgG-labeled dextran for 30 min. To develop the staining, sections were incubated in a freshly prepared mixture of diaminobenzidine (DAB) substrate in substrate solution for 5 min. Sections were then counterstained with hematoxylin and blued in Richard Allen Bluing Reagent™, dehydrated in a graded series of ethanols and xylene, and coverslipped. Slides were reviewed by light microscopy.

Rabbit Immunoglobulin Fraction (Normal) or non-specific IgG_1 monoclonal diluted with PBS was used as a negative control. Monoclonal antibodies against HSP60 (Clone LK-1; StressGen, Victoria, BC, Canada)(dilution = 1:500), HSP90 (Clone AC88; StressGen)(dilution = 1:4000), HSP40 (C-20; Santa Cruz Biotechnology, Inc.,Santa Cruz, CA))(dilution = 1:2000), and $p16^{INK4a}$ (Clone 16P07, NeoMarkers, Inc., Fremont, CA) (1:800), and a polyclonal antibody against HSP70 (StressGen)(dilution = 1:4000) were used as the primary antibodies.

Tissue expression of HSP proteins and p16 was based solely on the intensity of the immunohistochemical staining averaged over the lesional area. The intensity was graded as: 0, no expression (negative); 1, weak expression; 2, moderate expression 3, high expression. Some tissues were graded as 0–1, 1–2, or 2–3, reflecting some variability in the staining intensity within a tissue section.

2.3. Statistical analysis

For analyses, staining intensity was grouped as 0 to 1, 1–2 to 2, and 2–3 to 3. We used standard contingency table methods, using Pearson χ^2 test and Mantel extension test for trend (P_{Trend}) to test for statistical significance ($P < 0.05$), to assess possible univariate associations of HSP immunostaining intensity with severity of lesion. Finally, we stratified univariate associations of HSP immunostaining intensity with severity of lesion on inflammation status to control for its effects.

3. Results

The results of our study to correlate HSP expression with grade of cervical neoplasia are shown in the Table 1. The intensity of immunostaining for our positive control, $p16^{INK4a}$, strongly increased with increasing severity of the lesion ($P_{Trend} < 0.0005$). Similarly, we observed a strong tendency for increased immunostaining intensity with increasing lesion severity for HSP40, HSP60, and HSP70 ($P_{Trend} < 0.0005$). Among the CIN3 cases, 64%, 80%, 36%, and 16% were scored for the strongest staining for $p16^{INK4a}$, HSP40, HSP60, and HSP70, respectively. However, we did not observe this trend for HSP90 immunostaining ($P_{Trend} = 0.8$). All negative controls were negative for staining (data not shown).

We then restrict our analyses to those tissues that $p16^{INK4a}$ positive (tissue staining intensity > 1) to control for misclassification of histology and to restrict to those cases in which oncogenic HPV was expressed (n.b., we excluded the one case of normal histology that was $p16^{INK4A}$-positive for this analysis) (Table 2). Among the $p16^{INK4A}$-positives, HSP40 staining intensity was most strongly related to CIN3 compared to CIN1, with 85% of the CIN3 cases having the highest staining intensity compared to only 12.5% of the CIN1 cases ($P < 0.0005$). By comparison, HSP60 was only weakly related to having CIN3 ($P = 0.03$) and HSP70 was unrelated ($P = 0.4$).

Inflammation was associated with HSP40 ($P_{Trend} = 0.04$), HSP60 ($P_{Trend} = 0.001$), and $p16^{INK4a}$ ($P_{Trend} = 0.01$) immunostaining intensity but not with HSP70 and HSP90. However, stratification on inflammation status did not abrogate associations of HSP40 ($P_{Trend} = 0.001$), HSP60 ($P_{Trend} < 0.0005$), and HSP70 ($P_{Trend} < 0.0005$) immunostaining and severity of lesion.

Table 1
p16^{INK4a} (p16), HPS40, HSP60, HSP70, and HSP90 immunohistochemistry staining intensity for cervical tissues of different disease severity

Staining Intensity		Normal ($N=30$)		CIN1 ($N=32$)		CIN3 ($N=25$)		P^a	$P_{Trend}{}^b$
		N	%	*N*	%	*N*	%		
p16									
	0 or 1	29	96.7%	24	75.0%	5	20.0%	<0.0005	<0.0005
	1–2 or 2	1	3.3%	8	25.0%	4	16.0%		
	2–3 or 3	0	0.0%	0	0.0%	16	64.0%		
HSP40									
	0 or 1	4	13.3%	24	75.0%	0	0.0%	<0.0005	<0.0005
	1–2 or 2	26	86.7%	7	21.9%	5	20.0%		
	2–3 or 3	0	0.0%	1	3.1%	20	80.0%		
HSP60									
	0 or 1	23	76.7%	7	21.9%	6	24.0%	<0.0005	<0.0005
	1–2 or 2	7	23.3%	24	75.0%	10	40.0%		
	2–3 or 3	0	0.0%	1	3.1%	9	36.0%		
HSP70									
	0 or 1	24	80.0%	12	37.5%	4	16.0%	<0.0005	<0.0005
	1–2 or 2	6	20.0%	20	62.5%	17	68.0%		
	2–3 or 3	0	0.0%	0	0.0%	4	16.0%		
HSP90									
	0 or 1	29	96.7%	25	78.1%	25	100.0%	0.007	0.8
	1–2 or 2	1	3.3%	7	21.9%	0	0.0%		
	2–3 or 3	0	0.0%	0	0.0%	0	0.0%		

[a] Pearson χ^2.
[b] Mantel extension of the χ^2 test for trend.

HSP40 staining was diffuse throughout the normal and CIN1 tissue (Fig. 1a). By comparison, HSP60 and HSP70 staining, when positive, was primarily confined to basal layer in normal tissue (Fig. 1b) but was diffuse throughout CIN1 tissue.

An example of CIN3 case with strong HSP immunostaining is shown in the Fig. 2. Distinct tissue patterns of diffuse cytoplasmic immunostaining were observed for p16^{INK4a}. Diffuse HSP40 and HSP70 immunostaining was also observed in a similar tissue

Table 2
HPS40, HSP60, and HSP70 immunohistochemical staining intensity among p16INK4A-positive (staining >1) CIN1 and CIN3 tissues

Staining intensity		CIN1 ($N=8$)		CIN3 ($N=20$)		P^a
		N	%	*N*	%	
HSP40						
	0 or 1	6	75.0%	0	0.0%	<0.0005
	1–2 or 2	1	12.5%	3	15.0%	
	2–3 or 3	1	12.5%	17	85.0%	
HSP60						
	0 or 1	0	0.0%	5	25.0%	0.03
	1–2 or 2	8	100%	9	45.0%	
	2–3 or 3	0	0.0%	6	30.0%	
HSP70						
	0 or 1	1	12.5%	3	15.0%	0.4
	1–2 or 2	7	87.5%	13	65.0%	
	2–3 or 3	0	0.0%	4	20.0%	

[a] Pearson χ^2.

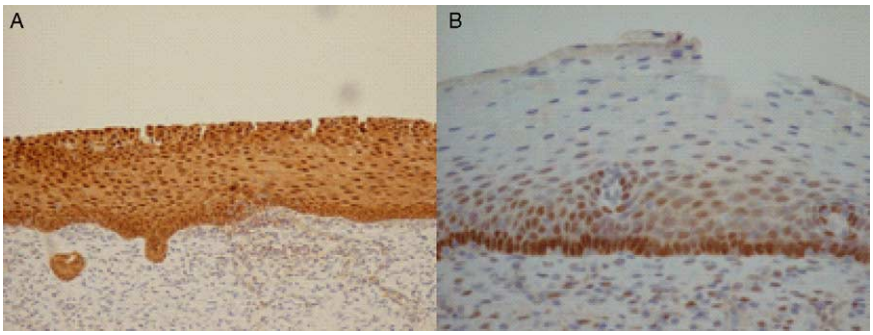

Fig. 1. Diffuse HSP40 immunohistochemical staining in normal tissue (a) and HSP70 immunohistochemical staining restricted to basal cells in normal tissue.

pattern as $p16^{INK4a}$ immunostaining. HSP60 immunostaining was observed in a similar tissue pattern as $p16^{INK4a}$ but cytoplasmic staining within cells was punctate rather than diffuse. In the CIN3 case shown, HSP90 immunostaining was weak and did not display any distinct pattern.

4. Discussion

In this study, we found that expression of HSP40, HSP60, and HSP70, but not HSP90, were increased with increasing severity of the cervical lesion, suggestive of stress responses to both the viral infection (CIN1) and an increased activation of the stress response throughout the progression of infection to precancer (CIN3). We confirm the previously reported correlations of HSP60 and HSP70 expression with lesion severity [13,14] and demonstrate that HSP40 likewise increases with lesion severity. Among those tissues with $p16^{INK4A}$ expression, a marker of HPV viral oncogenicity [21,22], HSP40 appeared to be most strongly related to progression from cervical HPV infection (CIN1) to precancer (CIN3).

We note that tissue patterns of HSP40 and HSP70 expression in CIN3 appeared remarkably similar to those for $p16^{INK4a}$, a biomarker of oncogenic HPV infection and CIN3 [21,22]. Increased expression of $p16^{INK4a}$ is a consequence of destabilization of retinoblastoma protein (rB) by oncogenic HPV E7 expression and activation of a negative transcriptional feedback loop that results in overexpression of $p16^{INK4a}$. HSP40, HSP70, and HSP90 have been hypothesized to interact with p53 in a regulatory manner [23]. Destabilization of p53 by oncogenic HPV E6 expression may result in up-regulation of HSPs in a manner analogous to $p16^{INK4a}$ and thus explain the similar distribution patterns. HSP40 and HSP70 are localized to both the cytoplasm and nucleus [2] and as a consequence, their expression appeared diffuse. HSP60 is localized to mitochondria [2] and thus possibly explaining the punctate appearance within cells. The lack of observed HSP90 expression in our study goes unexplained.

There were some limitations in our study that merit consideration. First, we used a qualitative measurement for assessing HSP expression and, as a result, there may be some misclassification. Thus, it is difficult to judge whether the distribution of HSP40 staining, with the higher percentage of negative or weakly staining intensity (0–1) in CIN1 versus normal, was real or artifact. A second limitation is that it is difficult to 'blind' these studies as histology was apparent during the review of staining intensity. A third limitation is that we did not have sufficient material for HPV DNA testing by PCR. Therefore, it is possible that some histologic normals were HPV DNA positive and that some CIN1, a poorly reproducible histologic diagnosis [20], were HPV DNA negative and represent an over-called diagnosis. We attempted to control for this later limitation using a secondary analysis that restricted to tissues that were $p16^{INK4A}$ positive. Together, the two aforementioned limitations would be expected to weaken trends of HSP expression with severity of lesion.

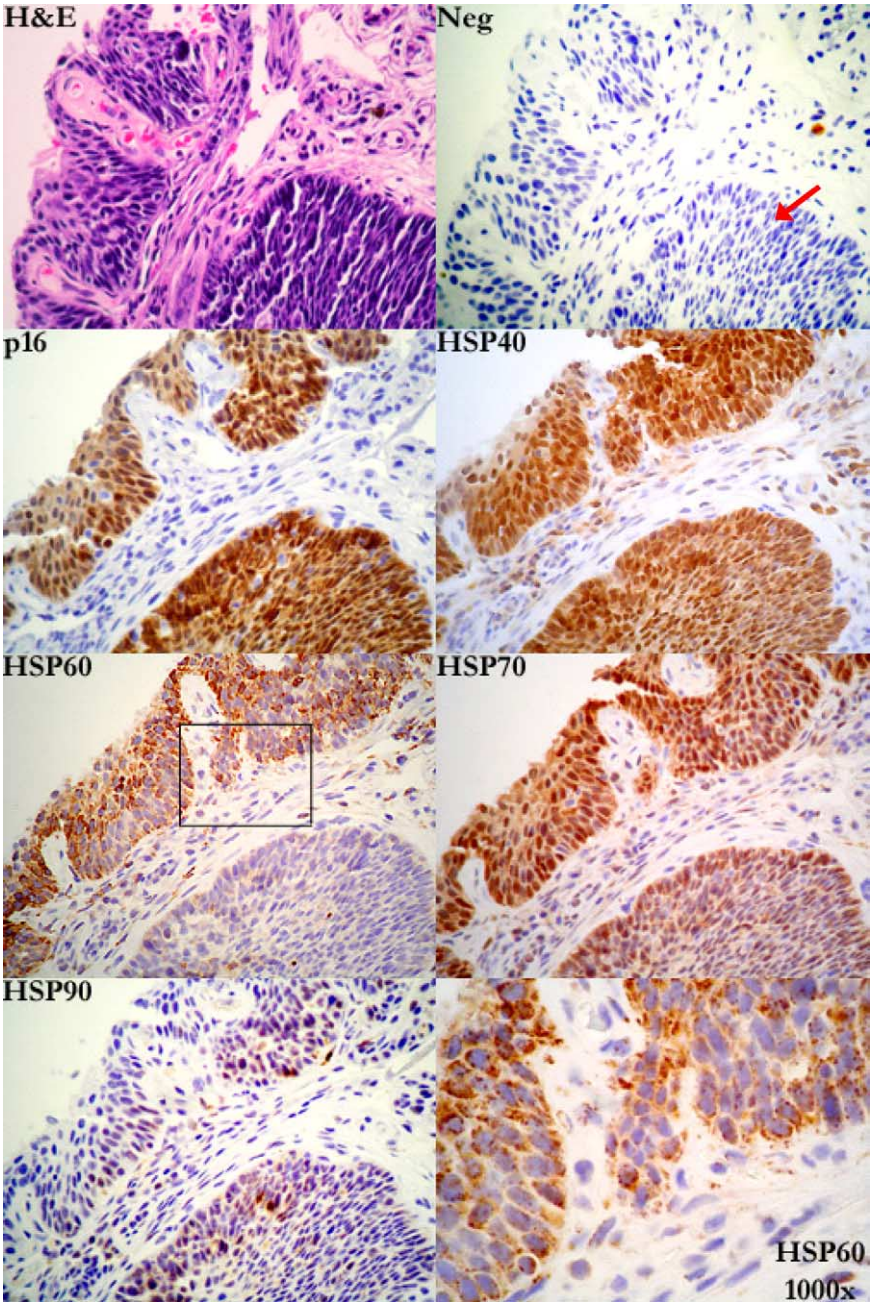

Fig. 2. A comparison of immunohistochemical staining patterns for $p16^{INK4a}$ (p16) (intensity = 3), HPS40 (intensity = 3), HSP60 (intensity = 2), HSP70 (intensity = 3), and HSP90 (intensity = 0) for a case of CIN3 using 400× magnification. The upper left panel shows the hematoxylin and eosin (H&E) stain and the upper right panel shows the negative (PBS control) staining. Other panels are label with the antigen specificity of the primary antibody. The boxed area in the HSP60 panel (third down on the left) highlights the area shown at 1000× in the lower right panel. The red arrow indicates a high-grade lesion invading an endocervical gland.

As mentioned, to control for oncogenic HPV DNA expression, we further restricted our analysis to the $p16^{INK4A}$ positive specimens. Comparing CIN3 to CIN1, we found that HSP40 expression was strongly associated with CIN3 compared to CIN1 whereas the HSP60 expression was only weakly associated and HSP70 expression was not associated with CIN3 compared to CIN1.

We also examined the effects of cervical inflammation on heat shock expression. Cervicitis does not appear to be associated with HPV infection but is associated with high-grade cervical neoplasia [24], which could be the result of co-infection with other sexually transmitted infections [25]. Thus, increased expression of HSPs, especially HSP60 and HSP70, could be a surrogate marker of cervicitis leading to precancer development rather than due to progression per se. Although inflammation was associated with several markers in this study, these associations of HSP immunostaining and severity of lesion remained robust after crude stratification by inflammation status. We therefore suggest that the HSP immunostaining cannot be fully explained as simply a marker of local inflammation.

In summary, we found some evidence for an up-regulation of HSPs in response to infection and early cervical carcinogenesis. We emphasize that this was a preliminary study and therefore further confirmation on a larger set of tissues is necessary with a larger panel of HSPs, which could be accomplished efficiently using tissue microarrays. In particular, it would be useful to assess HSPs in CIN2, which is likely a mix of CIN1 and CIN3 rather than a unique entity, and in cancer. Perhaps HSP40, which appeared strongly related to progression, may clarify the CIN2 diagnosis into CIN1 and CIN3 cases. Moreover, larger studies could examine whether there are expression differences of HSPs that correlate with different oncogenic potential of HPV types [16,17].

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